EVOLUTION OF LACTIC DEHYDROGENASES*

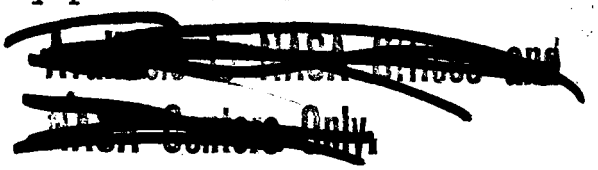
UNPUBLISHED PRELIMINARY DATA

By

A. C. Wilson, N. O. Kaplan, L. Levine, A. Pesce,† M. Reichlin‡ and W. S. Allison§§

Graduate Department of Biochemistry,
Brandeis University, Waltham, Massachusetts

(To appear in Federation Proceedings as part of the symposium on Biochemical Evolution)



There are various methods for comparing the structures of enzymes that catalyze the same reaction. The ideal method is to determine complete amino acid sequences. Cytochrome c and hemoglobin of several species have been examined by this method; the results and their evolutionary implications are discussed in the accompanying papers by Smith and Margoliash (20) and Hill (12) and elsewhere (e.g., 42, 45). Progress has been slow, however, because sequence determinations are tedious. Other, less ideal, methods of comparing enzyme structure have consequently been sought, rapid methods that would permit many enzymes to be surveyed quickly. We try to show here that much can be learned about enzyme evolution by means of these alternative methods.

The present paper should be viewed as a summary of some of our recent work along these lines. Many of the results to be mentioned have not been published before and will later be presented in detail in separate publications.

Most of our work has been done with lactic dehydrogenase (LDH). Two basically different types of LDH occur in the tissues of most animals (6,10,15,21,22,30). One type, which is inhibited strongly by high pyruvate concentrations, predominates in the heart and is called the heart LDH. The other type, less easily inhibited by pyruvate, predominates in many skeletal muscles and is called the muscle LDH. The two types seem to have different biological functions (7,43). The heart enzyme consists of 4 identical subunits, which we call H subunits. The muscle enzyme consists of 4 identical M subunits (Fig. 1). The two types of subunits, H and M, are very different in amino acid composition and fingerprint pattern (10,30), and there is genetic evidence that the two subunits are produced by two separate genes (34). Nevertheless, LDH's containing both H and M subunits can be formed. They are called hybrid enzymes. Three types of hybrid enzymes are possible, as indicated in Fig. 1, and all three are found in the tissues of many animals.

In our evolutionary studies, we have restricted attention to the two "pure" types, i.e., the heart type (H_4 LDH) and the muscle type (M_4 LDH). Twenty-five of these enzymes have been purified and crystallized in our laboratory from a variety of species, thanks largely to Mr. F. Stolzenbach. The species include six birds, three mammals, a reptile, two amphibians, two bony fish, a cartilaginous fish, a lamprey, a lobster and a bacterium (Table 1).

ULTRACENTRIFUGAL PROPERTIES

The sedimentation velocity of 16 of the pure LDH's has been measured

at several concentrations. The corrected sedimentation constants ($s^{o}_{20,w}$) are all close to 7.4 (Table 1). The sedimentation constants of the other nine enzymes, whose sedimentation was examined at only one concentration, would also be close to 7.4, if a similar concentration dependence were assumed.

Molecular weights have been calculated by the Ehrenberg procedure (8) for eight of the enzymes. The values are all close to 140,000 (Table 1). Since the bacterial enzyme has the same molecular weight as the M_4 and H_4 enzymes of animals, it appears that the size of LDH has stayed constant during evolution (29,30).

AMINO ACID COMPOSITIONS

Fourteen of the pure M_4 enzymes have been subjected to quantit ative amino acid analysis (29). The histidine content varies widely (Table 2), from about 70 residues per mole in the turkey enzyme to below 30 in the frog. Further, it is evident that closely related species have a similar content of histidine in their M_4 LDH. Animals belonging in the same vertebrate class are grouped in the table. Thus, all birds have a high histidine content. Both species of frog have a low content. The two mammals examined exhibit similar values. Note also that the caiman, which is a reptile related to the crocodile, has an M_4 enzyme with a histidine content intermediate between that of birds and other tetrapods. This is in agreement with the well known fact that birds have evolved from reptiles and, in particular, from a reptilian group allied to the crocodilians (11, 32, 38). In contrast to histidine, phenylalanine does not vary in content among M_4 LDH's (Table 2). Most amino acids fall between histidine and phenylalanine as regards variability. As was the case with histidine, so with the other variable amino acids, we find that, on the whole, closely related species have similar amino acid contents.

Eight crystalline H_4 enzymes have been analyzed for their amino acid contents (Table 3). Several amino acids vary significantly in content from species to species. For instance, the arginine content of the frog enzyme is lower than in birds and mammals. The isoleucine contents of the three mammalian enzymes are similar to each other and are distinct from those of birds and the frog. On the other hand, the lysine content is rather similar in all eight of the H_4 enzymes. The H_4 LDH data lead us to conclusions like those we draw from the M_4 LDH data, namely, that the amino acid composition varies considerably among various classes of vertebrates but that closely related species have similar compositions.

FINGERPRINT PATTERNS

Although amino acid analyses show that changes take place in the chemical structure of LDH's during evolution, such data cannot be expected to give a clear picture of the extent to which the amino acid sequences are changing. The fingerprint method has some advantages in this regard. Dr. T. Fondy of our laboratory recently began to examine the fingerprint patterns of several crystalline LDH's (9,10). The chicken H_4 enzyme yields about 30 ninhydrin-positive spots after trypsin treatment. The turkey enzyme gives a very similar pattern of spots but the patterns are not identical. The beef enzyme gives a very different pattern; at least 15 of the spots differ in chromatographic or electrophoretic properties from those found in the chicken enzyme. This indicates that there are many amino acid sequence differences between the beef and chicken H_4 enzymes.

The fingerprint patterns of four muscle LDH's have been compared with each other. The enzymes were from the chicken, bullfrog, halibut and dogfish, which represent four different classes of vertebrates. The four

patterns differ radically. We conclude that different classes of vertebrates have very different sequences in their muscle LDH's as well as in their heart LDH's, whereas closely related species, such as the chicken and turkey, have very similar sequences.

Dr. Fondy has isolated a peptide from the active site of LDH (9). He finds that the amino acid sequence in this peptide is virtually identical in the following LDH's: chicken and beef H_4 and chicken, bullfrog and dogfish M_4. The amino acid sequence may be more conservative in the active site peptide than in other parts of the LDH molecule (T. P. Fondy, unpublished observations).

COENZYME ANALOGS

In order to get the results discussed so far, pure crystalline enzymes were required. There are other methods of detecting evolutionary changes in the structure of enzymes, rapid methods that are applicable to crude tissue extracts. Coenzyme analogs provide such a method of detecting dehydrogenase evolution (16,17, 42). Table 4 presents a selection of our data on the catalytic properties of M_4 LDH of various vertebrate species. The acetylpyridine analog of DPN was compared with the natural coenzyme in this study. The chicken enzyme is seen to react as well with the analog as with the natural coenzyme under certain standard conditions of pH and lactate concentration. The M_4 enzyme of all other birds tested also reacts relatively well with the analog. Besides the birds referred to in the table, over 20 other species have been examined with similar results. In contrast to birds, other land animals have M_4 enzymes that react less well with the analog. The reptiles have an M_4 enzyme whose properties are intermediate between those of birds and other land animals (mammals and amphibians). This is in agreement with the histidine data presented above and with the fact that reptiles are the closest relatives of birds. Note also that coenzyme analogs permit the detection of differences between the turkey and chicken M_4 enzymes. The turkey and chicken belong to the same family (19,35) and can hybridize (28) with each other. Presumably the structural difference detected by the analog is a subtle one in this case. During recent years the M_4 and H_4 enzymes of over a hundred species have been examined by means of coenzyme analogs (16,17,42; Wilson and Kaplan, unpublished observations). The data support the thesis that the more closely related two species are, the more similar their LDH's are likely to be. A further conclusion is that the catalytic properties of LDH's are continually undergoing subtle changes during evolution.

TEMPERATURE STABILITY

Temperature stability varies among the LDH's of different species. In our survey of temperature stability, we have paid particular attention to the H_4 enzyme. Crude heart extracts can be used as sources of the H_4 enzyme for such studies. Under suitable conditions, for example, in the presence of buffered bovine serum albumin, the rate of enzyme inactivation at high temperature is identical for a crude extract and a pure H_4 enzyme from the same species and is independent of enzyme concentration. We find, as a general rule, that the H_4 enzymes of closely related species have rather similar temperature stabilities. Small, but significant differences often occur, however. For example, the bullfrog enzyme loses half of its activity in 20 minutes at 52° but under these conditions the leopard frog enzyme loses less than 5% of its activity (6). The temperature must be raised to 56° in order to inactivate the leopard frog enzyme at the same rate. In our experience with the H_4 enzymes of many species, measurements of rates of enzyme inactivation at carefully controlled temperatures often provide a useful method of differentiating between closely related species.

We also wish to draw attention to a large change in temperature stability. A great increase in temperature stability evidently took place at one point during vertebrate evolution. Among all the lower vertebrates that we have examined, including a diverse selection of fishes and amphibians, the inactivation temperature for H_4 LDH is in the neighborhood of 60° (Table 5). This is also true of the lower reptiles (turtles) and mammals examined. By contrast, all higher reptiles examined have an H_4 enzyme whose inactivation temperature is near 80°. Apparently, a great increase in temperature stability took place when the higher reptiles (Diapsida) were evolving from the lower reptiles (Anapsida).

The evolutionary line leading to mammals is presumed from anatomical and paleontological evidence to have come from anapsid reptiles before the higher reptiles evolved (32). Mammals have seemingly retained an H_4 enzyme whose temperature stability is of the lower vertebrate type.

Birds, in accordance with their evolution from higher reptiles, have highly temperature-stable H_4 enzymes, as a general rule (Table 5). However, in a few of the 24 bird orders that we have examined, a less stable H_4 enzyme occurs. These exceptional orders are not considered on anatomical grounds to be primitive among birds (26,37,41). Hence, it is likely that the relatively unstable H_4 enzyme found in these few orders has originated comparatively recently from the stable H_4 enzyme which is characteristic of primitive birds and higher reptiles. These tentative interpretations of the temperature stability data are summarized schematically in Fig. 2.

ELECTROPHORETIC MOBILITY

Electrophoretic mobility measurements can also be made with safety using crude extracts and a specific staining method for LDH. We have made a survey of this property in the H_4 LDH's of many species of birds and other vertebrates. Closely related species usually have rather similar mobilities, as shown in Table 5 and as was pointed out earlier by Vesell and Bearn (39). Frequently, however, closely related species exhibit significant differences. As an outstanding example, we cite the H_4 enzymes of the bullfrog and the leopard frog, which can easily be distinguished by electrophoretic methods (Table 5).

Large electrophoretic differences are more often noted when distant relatives are compared. For example, most mammals have a very rapidly migrating H_4 enzyme, whereas in most birds the mobility is very low (see Table 5 and ref. 39). Although there is considerable variation in electrophoretic mobility of the H_4 enzyme among species of a vertebrate class, certain general trends can be observed if many species of each class are investigated. We have measured the electrophoretic mobility of this enzyme, under standard conditions, in several species of each major vertebrate class (Table 5). The distance moved is in the range 5-10 cm for the great majority of fishes, amphibians and reptiles. Most mammals have an enzyme that moves 12-15 cm under these conditions. Evidently a significant increase in electrophoretic mobility took place when mammals evolved from reptiles.

By far the majority of birds have an H_4 LDH that moves only 1.5-3 cm under the standard conditions. Apparently, a reduction in electrophoretic mobility took place when birds evolved from reptiles. It is interesting to note, however, that certain birds, such as the ostrich, rhea and tinamou, which are termed paleognathous birds, have an H_4 enzyme whose mobility is like that of reptiles. These birds have long been considered on anatomical grounds to be related to each other and to be primitive among birds (11, 32, 38). If we assume that paleognathous birds are primitive among birds, and there has recently been some question about this (2), our data

imply that the reduction in electrophoretic mobility of H_4 LDH happened after paleognathous birds had evolved from reptiles and before the modern birds (neognathous birds) had diversified (Fig. 2).

MICRO-COMPLEMENT FIXATION

Overy 60 years have elapsed since Nuttall and others showed by immunological methods that serum proteins differ in structure from species to species (27). Many further studies of species differences have since been made, usually with antisera to protein mixtures from serum or egg white. Landsteiner (18) and Boyden (3) have summarized these studies, which show that the degree of immunological resemblance between proteins of different species is often a function of how closely related the species are. There have been relatively few studies of species differences with antisera directed to enzymes but they suffice to show that enzymes also exhibit the phenomenon of species specificity (4,5,44).

Immunological comparisons of enzymes, although not so fast as catalytic, electrophoretic and temperature-stability comparisons, can be done much faster than determinations of amino acid sequences, amino acid compositions or fingerprint comparisons. Highly purified enzymes are needed from each of the species to be compared by the last three methods. For immunological comparisons of enzymes, only the enzyme to be used as the immunizing antigen needs to be highly pure. Providing that this requirement is rigorously fulfilled, cross reactions with enzymes from other species can be measured unambiguously in crude extracts of an appropriate tissue.

In this article, we present results obtained with rabbit antisera directed toward several pure, crystalline enzymes (mainly lactic dehydrogenases) and certain other pure proteins. The method used to measure the antigen-antibody reactions was quantitative microcomplement fixation (40). It is a very sensitive method of measuring the uptake of complement that occurs when an antigen and an antibody interact and form a lattice-work. The method requires a hundred to a thousand times less antiserum and antigen than is required for conventional, quantitative immunological methods. Furthermore, microcomplement fixation is more sensitive to differences in protein structure than are conventional immunological methods.

To illustrate the sensitivity of the micro-C' F method, we present results obtained with rabbit antisera to human hemoglobin A_1. The amino acid sequence of A_1 is known to differ by only one amino acid residue from the sequence in hemoglobins S and C (14). Conventional immunological methods cannot distinguish these three hemoglobins with certainty. The micro-C'F method easily distinguishes S from A_1, as Fig. 3 shows. The top curves represent the reaction between A_1 and the antisera; the lower curves represent the reaction between S and the antisera. The three antisera also distinguish C from A_1 with equal efficiency (31) when the micro-C'F method is used.

To find out whether micro-complement fixation is always more sensitive to differences in protein structure, three other immune systems were investigated. The rabbit antisera used were (a) antihuman serum albumin, (b) anti-chicken ovalbumin and (c) anti-chicken H_4 LDH. Each antiserum was reacted with its homologous antigen and also with a related heterologous antigen (Table 6). Three methods were used to detect the reactions: (a) quantitative micro-C'F, (b) the quantitative C'F method of Mayer and others (25), which we term the macro-C' F method, and (c) the quantitative precipitin method. As measured by the last two methods, the heterologous proteins react about as well as the homologous antigens. By contrast, large differences were detected between the homologous and heterologous reactions by the micro-C'F method. It would seem that micro-C'F may always be more sensitive to dif-

ferences in protein structure than are conventional immunological methods.

Besides hemoglobins S and C, two other hemoglobins whose amino acid sequences are known have been examined by the micro-C'F method with an antiserum to hemoglobin A_1 (Table 7). S and C, as already noted, react less well than A_1. In order to observe a strong reaction with S or C the antiserum concentration has to be raised by a factor of 1.3. Hemoglobin A_2 is known to differ in sequence from A_1 by about 7-8 residues (14). A strong reaction with A_2 is observed if the antiserum concentration is raised by a factor of 2.0 (31). Hemoglobin F differs in amino acid sequence by about 40 residues from A_1 (33), and does not react strongly until the antiserum concentration is raised by a factor of more than 5. These results suggest that there is a correlation between the cross reactivity, as measured by the micro-C'F method, and the number of amino acid substitutions.

Because of this correlation, we have used the micro-C'F method to survey the evolution of lactic dehydrogenases and certain other enzymes. Fig. 4 shows some results obtained with an antiserum to pure, crystalline chicken H_4 LDH. In the top curve of Fig. 4a, the reaction between the antiserum and the pure enzyme is shown. If a crude extract of chicken heart is used an identical curve is obtained (not shown). The antiserum dilution used in this experiment was 1:30,000. At this antiserum dilution, the turkey H_4 LDH reacts poorly, as shown by the lower curve. In order to observe a strong reaction with the turkey enzyme, the antiserum concentration must be raised to 1:20,000 as the top curve of Fig. 4b shows. At this antiserum concentration, the bullfrog enzyme does not react. In order for the bullfrog enzyme to react strongly, a 12-fold increase in antiserum concentration (1:2500) must be employed as shown in Fig. 4c. As a measure of cross reactivity, we consequently use the relative concentration of antiserum required for a given percentage of C'F (by convention, 50% C'F, as measured at the peak of the C' F curve, i.e., at the equivalence zone). That is, we obtain a value for the ratio E/O, where E is the antiserum concentration required for 50% C'F with the heterologous antigen and O is the antiserum concentration required for 50% C'F with the homologous antigen. This value can be viewed as an immunological index of dissimilarity between a homologous and a heterologous antigen. The index of dissimilarity, measured in this way, has an experimental error of plus or minus 10%, for a given antiserum. When other antisera directed to the same antigen are used, slightly different values may be obtained. We have examined 10 different antisera to pure chicken H_4 LDH; the index of dissimilarity ranges from 1.1 to 1.7 for the turkey enzyme and from 6 to 19 for the frog enzyme.

Further results obtained with antisera to chicken heart LDH are shown in Table 8 (first column). The species whose H LDH's were examined form an approximate evolutionary series, ranging from the turkey, which is a close relative of the chicken, to the fishes which are remote relatives of the chicken. The H_4 LDH's were tested for reactivity with antisera to the chicken enzyme and the indices of dissimilarity calculated as described above. The turkey, duck, pigeon and ostrich enzymes react relatively well, the reptilian enzymes less well, the frog enzyme still less well, and the fish enzymes weakly, if at all.

We have done similar experiments with antisera to pure chicken M_4 LDH. Table 8 shows that these antisera reat well with the M_4 enzymes of other birds, less well with reptilian M_4 enzymes and still less well with the M_4 enzymes of the frog and fishes. It will be noted that the indices of dissimilarity agree semi-quantitatively with those which were obtained in the H_4 LDH immune system. This seems to imply that the average rate of change in immunological structure has been similar for the two enzymes during evolution. This implication would be consistent with the data on amino acid

composition and fingerprint patterns mentioned above.

We have also measured the rates of immunological change in other enzymes by means of the micro-C'F method. The following enzymes were purified and crystallized from the chicken and inje cted into rabbits: muscle triose-phosphate dehydrogenase (1), liver glutamic dehydrogenase, and muscle aldolase. The slow, major component of adult chicken hemoglobin was also isolated and injected. The resulting antisera were tested against the corresponding enzymes and hemoglobin component of the species listed in Table 8. The indices of dissimilarity increase from turkey to fish in much the same way for each immune system. The five enzymes examined and hemoglobin are a diverse selection of intracellular, biochemically active proteins. Yet their immunological structures change at rather similar rates during evolution. The rates are by no means identical but they certainly have the same order of magnitude.

Using the same approach, we have studied the evolution of M_4 LDH and triosephosphate dehydrogenase (TPD) in fishes. Pure M_4 LDH and TPD were prepared from the halibut (Hippoglossus), which is a higher teleost belonging to the flatfish order. Rabbit antisera directed to each of the enzymes were tested by the micro-C'F method against the corresponding enzymes of the various fishes listed in Table 9. These fishes are arranged in an approximate evolutionary series; close relatives of the halibut being near the top of the list and distant relatives near the bottom. It is apparent that, on the whole, TPD and muscle LDH have changed their immunological structures at similar rates during fish evolution.

The idea that the enzymes and other biochemically active proteins of a species tend to evolve in parallel is not based only on immunological evidence. Amino acid sequence data obtained with cytochrome c and hemoglobin support this view. In the case of man and the horse, the two species in which both proteins have been sequenced, there is a striking parallel. Human cytochrome c and hemoglobin (α-chain) each differ from their counterpart in the horse by 12 amino acid residues per 100 (23,24). The conclusion that cytochrome c and hemoglobin tend to evolve at nearly equal rates is also evident from Margoliash and Smith's recent summaries of cytochrome c sequences (20,36) in relation to Zuckerkandl and Pauling's calculations on the rate of hemoglobin evolution (45). In addition, Hill's recent fingerprinting work shows that the β-chains of hemoglobin have changed only a little faster than the α-chains have during primate evolution (12,13). Altogether, sequence data support the idea that the enzymes and other biochemically active proteins of a species tend to evolve at roughly similar rates. Further work will be necessary to ascertain the mechanism underlying the phenomenon.

SUMMARY AND CONCLUSIONS

Twenty-five lactic dehydrogenases have been purified and crystallized from various species. Several methods have been used to compare the structures of these enzymes, including determinations of molecular weight, amino acid composition and fingerprint pattern. Lactic dehydrogenases in crude extracts of many other species have been investigated by measurements of temperature stability, electrophoretic mobility and catalytic activity with coenzyme analogs.

The quantitative micro-complement fixation method has been used to compare the structures of hemoglobins and of several other proteins. The method is more sensitive to differences in protein structure than are conventional, quantitative immunological methods. Single amino acid differences

aldolase,

among hemoglobins are easily detected by this method. The method also provides an approximate measure of the degree of structural resemblance between various hemoglobins of known sequence. We have used micro-complement fixation to examine rates of protein evolution. The proteins studied were H_4 lactic dehydrogenase, M_4 lactic dehydrogenase, triosephosphate dehydrogenase, glutamic dehydrogenase and hemoglobin. The results suggest that, in a given species, all these proteins tend to change in immunological structure at similar rates.

ACKNOWLEDGMENTS

We thank the many people who have kindly supplied specimens: W. Drury and J. Baird, Massachusetts Audubon Society; C. Gandal, W. Conway and H. Dowling, Bronx Zoo (New York Zoological Society); C. Sibley, Cornell University; R. Orr, California Academy of Sciences; E. Herald, Steinhart Aquarium; M. Mathews, La Rabida-University of Chicago Institute; M. Goodman, Wayne State University; W. Gosline, University of Hawaii; S. Tinker, Honolulu Aquarium; E. Gould and M. Steinberg, Bureau of Commercial Fisheries, Gloucester; D. Jensen, Scripps Institution of Oceanography; C. Huntington, Bowdoin College; J. King and D. Farner, Washington State University. We also thank Mrs. G. B. Kitto and the Misses N. Grimes, E. Wasserman, L. Dillahunt, J. Barber, J. Eaton, A. C. King and C. Barber for experimental assistance, Mr. F. Stolzenbach for purifying many enzymes, and Mr. F. Castillo for performing the amino acid analyses.

References

1. Allison, W.S. and N.O. Kaplan. J. Biol. Chem. 239: 0000, 1964.
2. Bock, W.J. Proc. Intern. Ornithol. Congr. 13: 39, 1963.
3. Boyden, A. In: Serological and Biochemical Comparisons of Proteins, edited by W.A. Cole. New Brunswick, N.J.: Rutgers University Press, 1958, p. 3.
4. Brown, R.K., B.C. Tacey and C.B. Anfinsen. Biochim. Biophys. Acta 39: 528, 1960.
5. Bueding, E. Federation Proc. 21: 1039, 1962.
6. Cahn, R.D., N.O. Kaplan, L. Levine and E. Zwilling. Science 136: 962,1962.
7. Dawson, D.M., T.L. Goodfriend and N.O. Kaplan. Science 143: 929, 1964.
8. Ehrenberg, A. Acta Chem. Scand. 11: 1257, 1957.
9. Fondy, T.P. and J. Everse. Federation Proc. 23: 424, 1964.
10. Fondy, T.P., A. Pesce, F. Stolzenbach, I. Freedberg and N. O. Kaplan. Biochemistry 3: 522, 1964.
11. Heilmann, G. The Origin of Birds. New York: D. Appleton, 1926.
12. Hill, R.L. Federation Proc. 00: 0000, 1964.
13. Hill, R.L., J. Buettner-Janusch and V. Buettner-Janusch. Proc. Nat. Acad. Sci. U.S. 50: 885, 1963.
14. Ingram, V.I. The Hemoglobins in Genetics and Evolution. New York: Columbia University Press, 1963.
15. Kaplan, N.O. Bacteriol. Rev. 27: 155, 1963.
16. Kaplan, N.O. and M.M. Ciotti. Ann. N.Y. Acad. Sci. 94: 701,1961.
17. Kaplan, N.O., M.M. Ciotti, M. Hamolsky and R.E. Bieber. Science 131: 392, 1960.
18. Landsteiner, K. The Specificity of Serological Reactions. Cambridge, Mass. Harvard Univ. Press, 1945.
19. Mainardi, D. Proc. Intern. Ornithol. Congr. 13: 103, 1963.
20. Margoliash, E. Proc. Nat. Acad. Sci. U.S. 50: 672, 1963.
21. Markert, C.L. Science 140:1329, 1963.
22. Markert, C.L. and H. Ursprung. Develop. Biol. 5: 363, 1962.
23. Matsubara, H. and E.L. Smith. J. Biol. Chem. 238: 2732,1963.
24. Matsuda, G., R. Gehring-Muller and G. Braunitzer. Biochem. Zeit. 338: 669, 1963.
25. Mayer, M.M., A.G. Osler, O.G. Bier and M. Heidelberger. J. Immunol. 59: 195, 1948.
26. Mayr, E. and D. Amadon. Amer. Museum Novit., no. 1496, 42 p., 1951.
27. Nuttall, G.H.F. Blood Immunity and Blood Relationships. Cambridge Univ. Press, 1904.
28. Olsen, M.W. J. Hered. 51: 69, 1960.
29. Pesce, A. Ph.D. Dissertation. Brandeis University, 1964.
30. Pesce, A., R.H. McKay, F. Stolzenbach, R.D. Cahn and N.O. Kaplan. J. Biol. Chem. 239: 0000, 1964.
31. Reichlin, M., M. Hay and L. Levine. Immunochemistry 1: 21, 1964.
32. Romer, A.S. Vertebrate Paleontology. Univ. of Chicago Press, 1945.
33. Schroeder, W.A., J.R. Shelton, J.B. Shelton and J. Cormick. Proc. Nat. Acad. Sci. U.S. 48: 284, 1962.
34. Shaw, C.R. and E. Barto. Proc. Nat. Acad. Sci. U.S. 50: 211,1963.
35. Sibley, C.G. Ibis. 102: 215, 1960.
36. Smith, E.L. and E. Margoliash. Federation Proc. 00: 0000, 1964.
37. Storer, R.W. In: Biology and Comparative Physiology of Birds, vol. 1, edited by A. J. Marshall. New York: Academic Press,1960, p. 57.
38. Swinton, W.E. Ibid., p. 1.
39. Vesell, E.S. and A. G. Bearn. J. Gen. Physiol. 45: 553, 1962.
40. Wasserman, E. and L. Levine. J. Immunol. 87: 290, 1961.
41. Wetmore, A. Smithson. Misc. Coll. 139: 1, 1960.
42. Wilson, A.C. and N.O. Kaplan. In: Taxonomic Biochemistry and Serology, edited by C.A. Leone. New York: Ronald, 1964, p. 321.
43. Wilson, A.C., R.D. Cahn and N.O. Kaplan. Nature 197: 331, 1963.
44. Yunis, A.A. and E.G. Krebs. J. Biol. Chem. 237: 34, 1962.
45. Zuckerkandl, E. and L. Pauling. In: Horizons in Biochemistry, edited by M. Kasha and B. Pullman. New York: Academic Press, 1962, p. 189.

TABLE 1. Sources and Ultracentrifugal Properties of Lactic Dehydrogenases

Taxonomic Group	Type of LDH	$S^{o}_{20,w}$	Molecular Weight
Chordata (Vertebrata)			
Mammalia			
Man	H	7.46	146,000
Domestic cattle	H	7.45	131,000
	M	7.32	153,000
Laboratory rabbit	H		
	M	7.23	
Aves			
Domestic chicken	H	7.31	151,000
	M	7.33	140,000
Pheasant, *Phasianus colchicus*	H		
	M	7.69	
Domestic turkey	H	7.49	
	M	7.52	
Ostrich, *Struthio camelus*	H		
	M		
Rhea, *Rhea pennata*	M		
Domestic duck	M		
Reptilia			
Caiman, *Caiman crocodilus*	M		
Amphibia			
Leopard frog, *Rana pipiens*	H	7.70	
	M		
Bullfrog, *Rana catesbiana*	M	7.56	154,000
Osteichthyes			
White sturgeon, *Acipenser transmontanus*	M		
Pacific halibut, *Hippoglossus stenolepis*	M	7.47	141,000
Chondrichthyes			
Spiny dogfish, *Squalus acanthias*	M	7.54	
Cyclostomata			
Sea lamprey, *Petromyzon marinus*	M	7.36	
Arthropoda			
Maine lobster, *Homarus americanus*	M	7.25	
Bacteria			
Lactobacillus plantarum (ATCC 8041)		7.50	153,000

TABLE 2. Amino Acid Composition of M_4 LDH

Species	Histidine*	Phenylalanine*
Chicken	63	27
Pheasant	61	29
Turkey	73	24
Duck	57	27
Ostrich	65	26
Rhea	66	28
Caiman	46	26
Cow	33	29
Rabbit	41	26
Leopard frog	26	28
Bullfrog	29	26
Halibut	49	29
Dogfish	42	26
Lamprey	41	24

*Residues per mole of enzyme.

TABLE 3. Amino Acid Composition of H_4 LDH

Species	Arginine*	Isoleucine*	Lysine*
Chicken	35	66	99
Pheasant	38	67	97
Turkey	34	69	87
Ostrich	36	68	95
Cow	34	85	96
Rabbit	33	84	97
Man	30	88	96
Leopard frog	21	65	95

*Residues per mole of enzyme.

TABLE 4. Reactivity of M_4 LDH with the Coenzyme Analog AcPyDPN

Species	$\frac{\text{AcPyDPN}}{\text{DPN}}$*
Chicken	1.1
Turkey	2.0
Duck	0.8
Caiman	0.7
Painted turtle	0.6
Bullfrog	0.28
Leopard frog	0.35
Green frog	0.24
Cow	0.28
Man	0.18

*The relative rates of reaction with AcPyDPN and DPN, measured with lithium L-lactate (1.3×10^{-2} M) as substrate, are expressed by the ratio AcPyDPN/DPN. Most of the results were obtained with pure M_4 LDH's, but in several cases we used a crude extract of an appropriate tissue; electrophoretic and immunological criteria showed that at least 80% of the LDH in the tissue extracts used was M_4 LDH. Whenever such a crude extract was compared with pure M_4 LDH from the same species, identical coenzyme analog ratios were obtained.

TABLE 5. Temperature Stability and Electrophoretic Mobility of H_4 LDH

Taxonomic Group	Inactivation Temperature*	Electrophoretic Mobility†
Neognathous Birds‡		
Passeriformes (6/67)	74	1.8
Piciformes (3/7)	79	2.8
Coraciiformes (2/9)	80	2.2
Trogoniformes (1/1)	68	0.2
Apodiformes (2/3)	--	2.2
Caprimulgiformes (1/5)	--	0.2
Strigiformes (1/2)	68	2.1
Cuculiformes (2/2)	80	2.4
Psittaciformes (1/1)	67	2.9
Columbiformes (2/2)	--	2.2
Charadriiformes (6/15)	77	1.5
Gruiformes (2/12)	63	2.2
Galliformes (1/3)	77	2.8
Falconiformes (3/5)	78	2.1
Anseriformes (1/2)	76	2.1
Ciconiiformes (1/7)	79	2.1
Pelecaniformes (3/5)	80	1.7
Procellariiformes (2/4)	76	1.5
Podicipediformes (1/1)	79	1.5
Gaviiformes (1/1)	80	1.8
Sphenisciformes (1/1)	79	2.0
Paleognathous birds		
Tinamiformes (1/1)	80	6.6
Rheiformes (1/1)	79	6.8
Struthioniformes (1/1)	80	6.6
Higher Reptiles		
Caiman, *Caiman crocodilus*	76	5.1
Lizard, *Iguana iguana*	82	5.6
Lizard, *Varanus flavicens*	85	2.5
Snake, *Natrix* species	80	6.3
Snake, *Crotalus atrox*	80	6.3
Snake, *Constrictor constrictor*	77	5.3
Lower Reptiles		
Snapping turtle, *Chelydra serpentis*	58	--
Painted turtle, *Chrysemys picta*	52	5.7
Cooter turtle, *Pseudemys scripta*	--	6.3
Soft-shell turtle, *Trionyx ferox*	<60	5.2
Mammals		
Man	65	15
Domestic cow	61	11
Domestic pig	61	12
Laboratory rabbit	65	12
Laboratory mouse	60	11
Laboratory rat	60	12
Squirrel, *Sciurus carolinensis*	68	11
European hedgehog, *Erinaceus europaeus*	65	11
Short-tail shrew, *Blarina brevicauda*	66	7
Opossum, *Didelphis virginiana*	--	15
Kangaroo, *Macropus robustus*	69	15
Amphibians		
Bullfrog, *Rana catesbiana*	52	10

Taxonomic Group	Inactivation Temperature*	Electrophoretic Mobility†
Leopard frog, *Rana pipiens*	56	3.4
Toad, *Bufo marinus*	65	7.4
Congo eel, *Amphiuma tridactylum*	68	11
Bony fish		
Sturgeon, *Acipenser transmontanus*	62	6.9
Haddock, *Melanogrammus aeglefinus*	63	7.8
Mackerel, *Scomber scrombrus*	60	6.1
Cartilaginous fish		
Seven-gill shark, *Notorhynchus maculatum*	66	5.0
Spiny dogfish, *Squalus acanthias*	64	4.1
Chimaera, *Hydrolagus collei*	<60	7.0
Cyclostomes		
Lamprey, *Petromyzon marinus*	δ	δ
Hagfish, *Eptatretus stouti*	--	4.8

* Temperature (°C) required for 50% inactivation in 20 minutes.

† Distance (cm.) moved during horizontal starch-gel electrophoresis at pH 7 for 16 hours at 10 volts/cm.

‡ So many birds have been studied that we have averaged the data for each order. The neognathous orders are arranged in a sequence, those orders near the bottom of the list being considered by ornithological authorities to be more primitive than those near the top of the list. In parentheses, we record the number of families examined as a fraction of the total number of families in the order.

δ The lamprey has no H_4 LDH in the heart; all lamprey tissues examined contain only M_4 LDH, as judged by immunological, electrophoretic and catalytic criteria.

TABLE 6. Comparison of Sensitivity of Immunological Methods

Antiserum	Heterologous antigen	Cross reaction*		
		Micro-complement fixation	Macro-complement fixation	Quantitative precipitin reaction
Anti-human hemoglobin A_1	Human hemoglobin S	41	86	100
Anti-human serum albumin	Chimpanzee serum albumin	52	97	83
Anti-chicken ovalbumin	Turkey ovalbumin	3	89	87
Anti-chicken H_4 LDH	Turkey H_4 LDH	32	91	89

* The heterologous reaction is expressed as a percentage of the homologous reaction. Data taken from Reichlin, Hay and Levine (31) and Reichlin, Wilson and Levine, unpublished experiments.

TABLE 7. Micro-complement Fixation by Antiserum to Hemoglobin A_1

Hemoglobin	No. of Amino Acid Substitutions	Antiserum Concentration for 50% C' Fixation*
A_1	0	1.0
S	1	1.3
C	1	1.3
A_2	7-8	2.0
F	40	>5

* Data obtained with the antiserum no. 272B4 used by Reichlin, Hay and Levine (31).

TABLE 8. Micro-complement Fixation With Antisera to Pure Chicken Proteins

Species	Antiserum concentration required for 50% C' fixation*					
	H_4 LDH	M_4 LDH	TPD	GDH	Aldolase	Hemoglobin
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Turkey	1.4	1.2	1.0	1.0	1.0	1.0
Duck	1.5	4.3	1.2	1.2	-	2.2
Pigeon	2.3	2.0	1.3	1.3	-	3.6
Ostrich	1.9	3.1	1.3	1.4	5.0	-
Caiman	3.3	4.2	3.8	4.0	6.5	-
Painted turtle	4.0	5.2	4.2	4.0	-	6.5
Bullfrog	14	40	30	19	18	-
Sturgeon	80	20	12	-	25	-
Halibut	†	>200	>50	-	>100	-
Dogfish	>100	>200	>50	-	-	-
Lamprey	†	>30	>50	-	-	-
Hagfish	>100	>100	-	-	-	-

* The data presented are based on the use of several anti-chicken H_4 LDH sera, two anti-chicken M_4 LDH sera and one each of the other anti-chicken protein sera.

† Halibut and lamprey tissues contain no detectable H_4 LDH.

TABLE 9. Micro-complement Fixation by Antisera to Halibut Enzymes

Species	Antiserum concentration for 50% C' fixation: TPD	M_4 LDH
BONY FISHES		
Teleostei		
Heterosomata		
Pacific halibut, *Hippoglossus stenolepis*	1.0	1.0
Petrale sole, *Eopsetta jordani*	1.3	1.1
Commercial flounder (Boston)	1.3	1.1
Commercial sole (Boston)	1.3	1.1
Perciformes		
Scorpion fish, *Scorpaenopsis gibbosa*	2.0	1.2
Mackerel, *Scomber scombrus*	1.8	1.5
Beryciformes		
Squirrel fish, *Holocentrus ensifer*	1.8	1.3
Ostariophysi		
Carp, *Cyprinus carpio*	16	1.6
Brown bullhead catfish, *Ictalurus nebulosus*	18	1.7
Apodes		
Conger eel, *Conger marginatus*	17	2.0
Moray eel, *Gymnothorax hepaticus*	20	4.0
Moray eel, *Gymnothorax flavimarginatus*	20	4.2
Isospondyli		
Brook trout, *Salvelinus fontinalis*	6.0	1.6
Commercial salmon (Boston)	6.0	1.6
Commercial smelt, *Osmerus mordax*	4.1	3.2
Chain pickerel, *Esox niger*	11	6.0
Holostei		
Gar pike, *Lepisosteus spatula*	17	20
Palaeoniscoidei		
Sturgeon, *Acipenser transmontanus*	67	35
Dipnoi		
African lungfish, *Protopterus* species	>200	100
CARTILAGINOUS FISHES		
Spiny dogfish, *Squalus scanthias*	>200	>200
JAWLESS FISHES		
Lamprey, *Petromyzon marinus*	>200	>200

Footnotes

* Publication No. 000 from the Graduate Department of Biochemistry, Brandeis University. Supported in part by research grants from the National Aeronautics and Space Administration (NSG-375), the Air Force Office of Scientific Research (AF-AFOSR-214-63), the American Cancer Society (P77F) and the National Institutes of Health (A1-01940 and AI-02792).

† Public Health Service Predoctoral Fellow (GPM-17680).

‡ Postdoctoral Fellow, Public Health Service Trainee (grant T1GM-212).

§ Public Health Service Predoctoral Fellow (GM-12,63813).

FIGURE LEGENDS

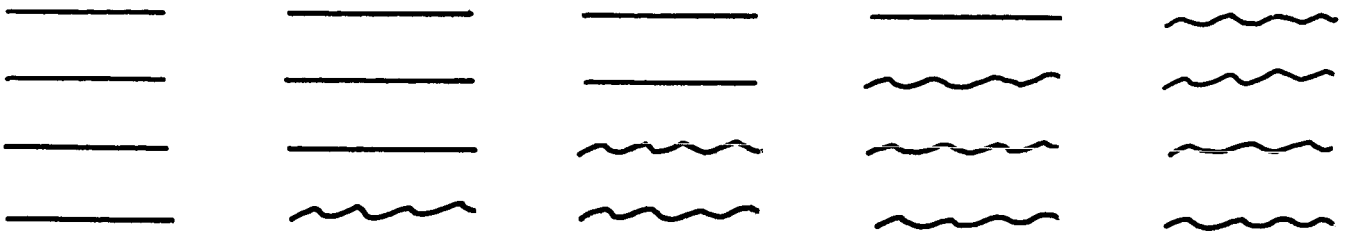
Fig. 1. Schematic representation of the five forms of lactic dehydrogenase.

Fig. 2. Temperature stability and electrophoretic mobility of H_4 lactic dehydrogenase during vertebrate evolution. A tentative and simplified schematic representation of the data shown in Table 5.

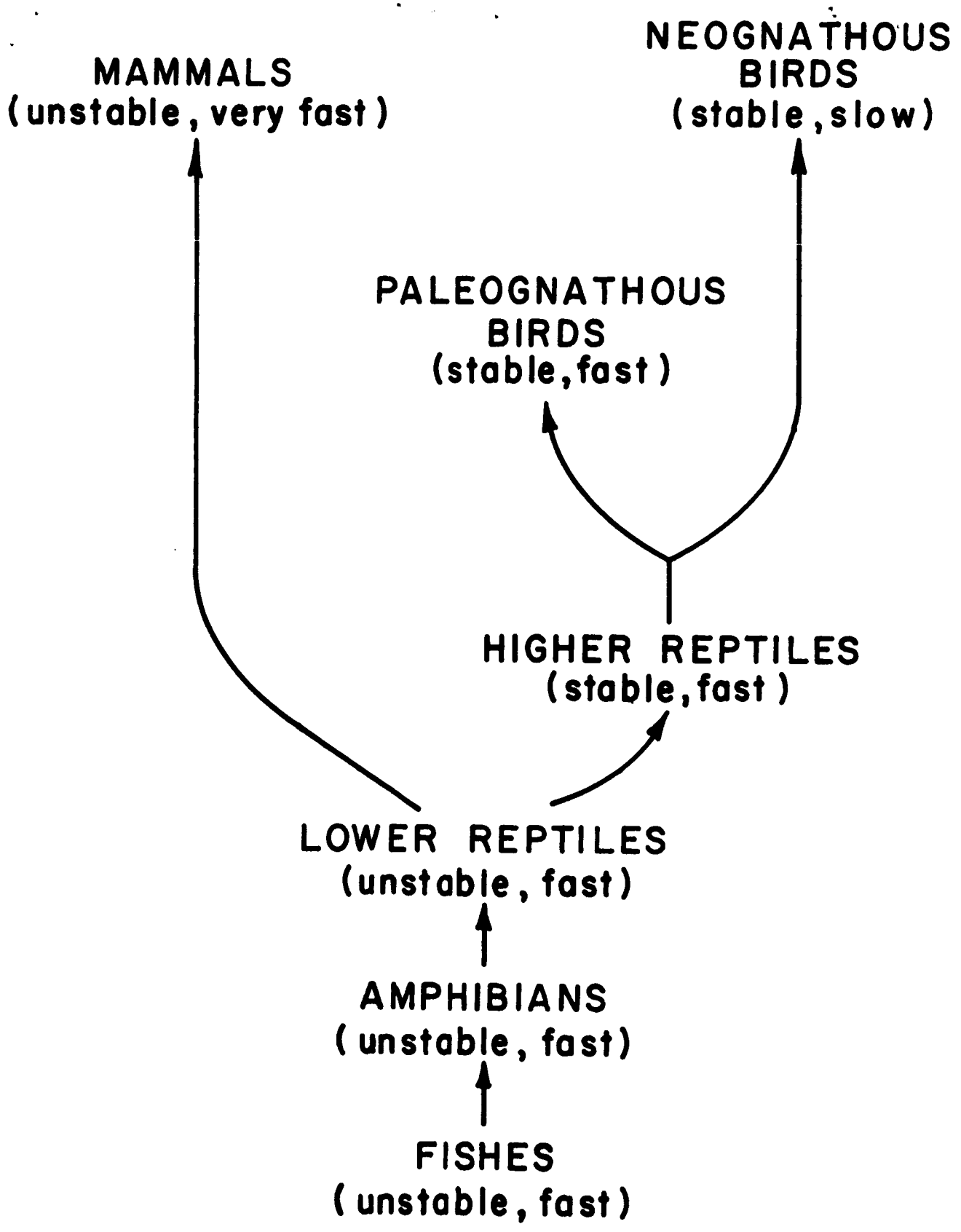
Fig. 3. Micro-complement fixation with antisera to hemoglobin A_1 by hemoglobin A_1 (●) and hemoglobin S (○). From Ref. 31.

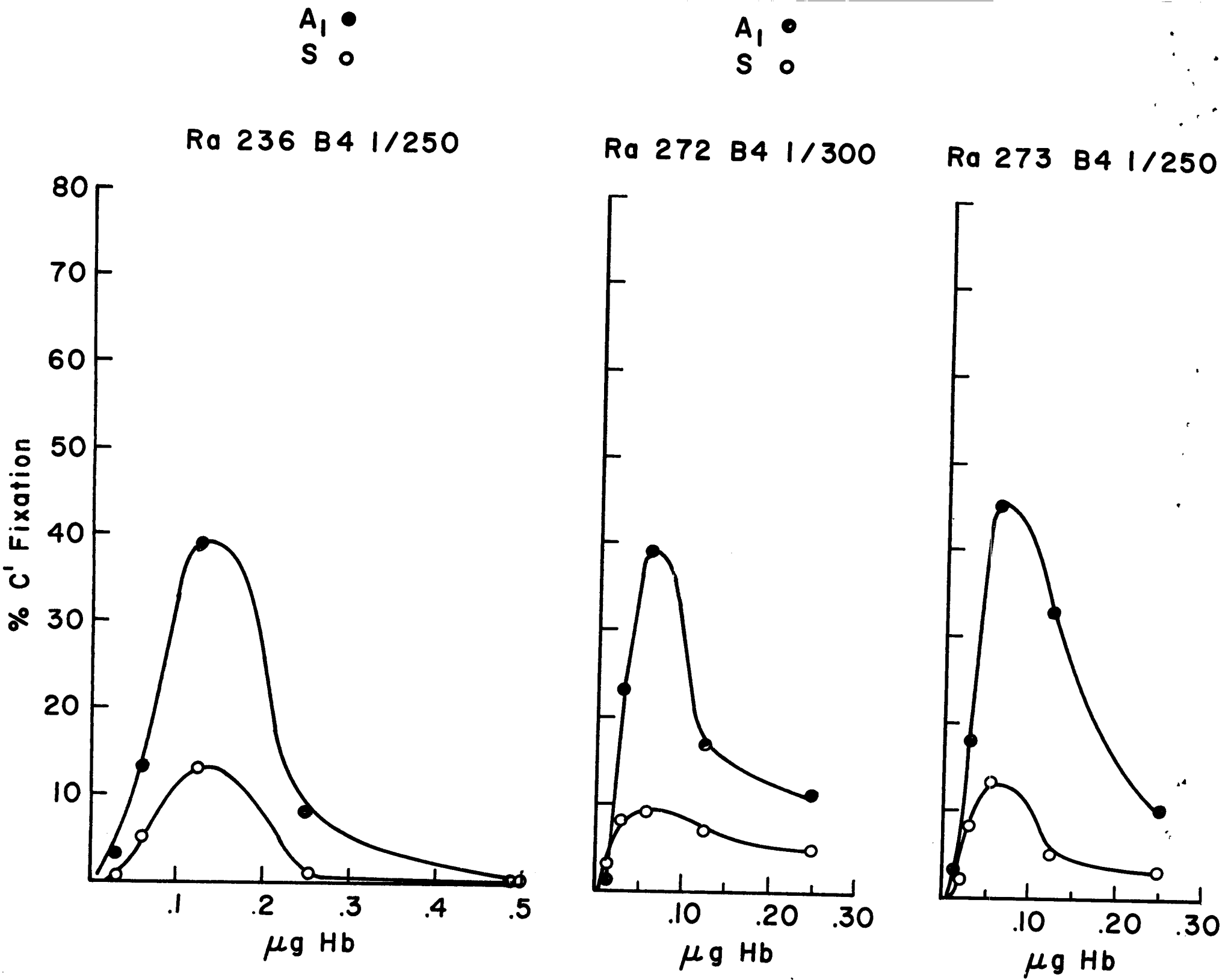
Fig. 4. Micro-complement fixation with antiserum to pure chicken H_4 lactic dehydrogenase by pure chicken H_4 lactic dehydrogenase, pure turkey H_4 lactic dehydrogenase and a crude extract of bullfrog heart. Crude extracts of chicken and turkey hearts gave complement fixation curves identical with those given by the pure enzymes. The antiserum dilutions employed were (a) 1:30,000, (b) 1:20,000, and (c) 1:2,500.

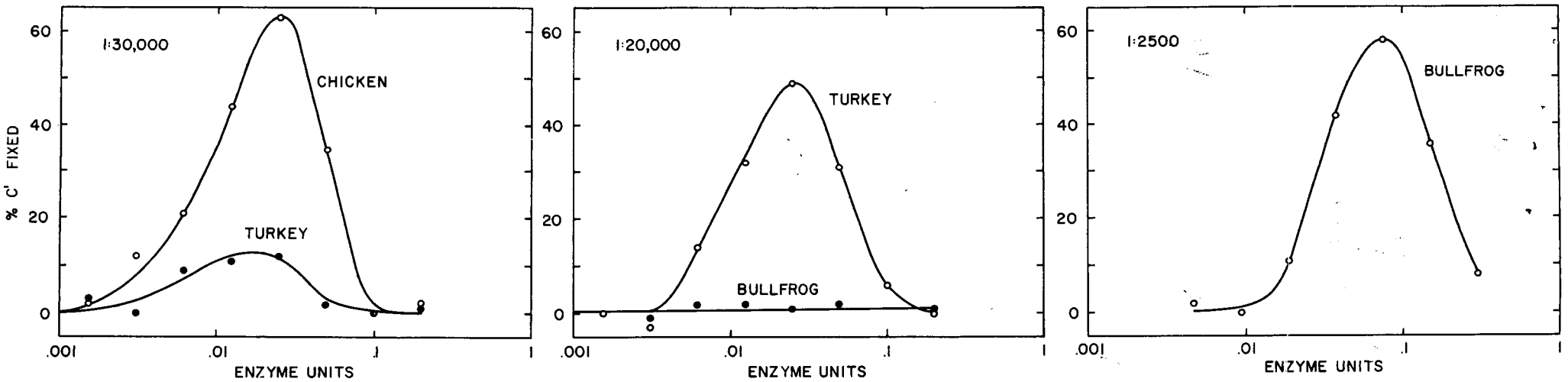
TYPES OF LDH



HHHH HHHM HHMM HMMM MMMM


MAMMALS
(unstable, very fast)
NEOGNATHOUS
BIRDS
(stable, slow)
PALEOGNATHOUS
BIRDS
(stable, fast)
HIGHER REPTILES
(stable, fast)
LOWER REPTILES
(unstable, fast)
AMPHIBIANS
(unstable, fast)
FISHES
(unstable, fast)


A1 ●
S o
Ra 236 B4 1/250
80
70
60
50
40
30
20
10
% C' Fixation
.1
.2
.3
.4
.5
μg Hb
A1 ●
S o
Ra 272 B4 1/300
.10
.20
.30
μg Hb
Ra 273 B4 1/250
.10
.20
.30
μg Hb


1:30,000
CHICKEN
TURKEY
% C' FIXED
60
40
20
0
.001
.01
.1
1
ENZYME UNITS
1:20,000
TURKEY
BULLFROG
1:2500
BULLFROG